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Inactivation of $NADP^+$-dependent Isocitrate Dehydrogenase by Lipid Peroxidation Products

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Membrane lipid peroxidation processes yield products that may react with proteins to cause oxidative modification. Recently, we demonstrated that the control of cytosolic and mitochondrial redox balance and oxidative damage is one of the primary functions of $NADP^+$-dependent isocitrate dehydrogenase (ICDH) through to supply NADPH for antioxidant systems. When exposed to lipid peroxidation products, such as malondialdehyde (MDA), 4-hydroxynonenal (HNE) and lipid hydroperoxide, ICDH was susceptible to oxidative damage, which was indicated by the loss of activity and the formation of carbonyl groups. The structural alterations of modified enzymes were indicated by the change in thermal stability, intrinsic tryptophan fluorescence and binding of the hydrophobic probe 8-anilino 1-napthalene sulfonic acid. Upon exposure to 2,2′-azobis(2-amidinopropane) hydrochloride (AAPH), which induces lipid peroxidation in membrane, a significant decrease in both cytosolic and mitochondrial ICDH activities were observed in U937 cells. Using immunoprecipitation and immunoblotting, we were able to isolate and positively identify HNE adduct in mitochondrial ICDH from AAPH-treated U937 cells. The lipid peroxidation-mediated damage to ICDH may result in the perturbation of the cellular antioxidant defense mechanisms and subsequently lead to a pro-oxidant condition.

Keywords: Lipid peroxidation; Isocitrate dehydrogenase; NADPH; Conformational change; U937 cell

INTRODUCTION

Reactive oxygen species (ROS), such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($^{\cdot}OH$), are generated *in vivo* from the incomplete reaction of oxygen during aerobic metabolism, stimulated host phagocytes, or from exposure to environmental agents such as radiation and redox cycling agents. These oxygen species can cause widespread damage to biological macromolecules leading to lipid peroxidation, protein oxidation, and DNA base modification and strand breaks.[1,2] Oxidative stress and ROS-mediated cell damage have been implicated in aging and a variety of human diseases including alcohol-mediated organ damage, various forms of neurodegenerative diseases, many types of cancers, cardiovascular diseases, lung diseases, and UV-mediated skin diseases.[3]

In biological membranes, lipid peroxidation is frequently a consequence of free radical attack. The peroxidation of unsaturated fatty acids of cells produces many reactive species such as free radicals, hydroperoxides, and carbonyl compounds, which may cause damage to proteins and DNA.[1] It has also been assumed that the decomposition of hydroperoxides mediated by catalytic transition metal ions may form much more toxic breakdown products such as alkoxy radicals ($RO^{\cdot}$), peroxy radicals ($ROO^{\cdot}$), $^{\cdot}OH$, and reactive aldehydes, including malondialdehyde (MDA) and 4-hydroxynonenal (HNE).[4,5] It is possible that in complex biological systems, oxygen free radicals and reactive aldehydes may cause protein and DNA damage indirectly by initiating lipid peroxidation, since polyunsaturated side chains of membrane lipids are especially susceptible to free radical-initiated oxidation.[6]

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Biological systems have evolved an effective and complicated network of defense mechanisms which enable cells to cope with lethal oxidative environments. These defense mechanisms involve antioxidant enzymes, such as superoxide dismutases (SOD), which catalyze the dismutation of O_2^- to H_2O_2 and O_2,[7] catalase, and peroxidases which remove hydrogen peroxide and hydroperoxides.[8] Glucose 6-phosphate dehydrogenase, which is a key enzyme for the generation of NADPH, and glutathione reductase, which is involved in the regeneration of reduced glutathione (GSH), are also considered as essential antioxidant enzymes.[9] These enzymes normally act as a team, thus SOD protects catalase and peroxidase against inhibition by O_2^-,[10] while catalase and peroxidase provide protection for SOD against inactivation by H_2O_2.[11] The damage brought about by oxidative stress is expected to be exacerbated if the antioxidant enzymes themselves are damaged and inactivated by such events.[12]

The isocitrate dehydrogenases (ICDHs; EC1.1.1.41 and EC1.1.1.42) catalyze oxidative decarboxylation of isocitrate to α-ketoglutarate and require either NAD^+ or $NADP^+$, producing NADH and NADPH, respectively.[13] NADPH is an essential reducing equivalent for the regeneration of reduced GSH by glutathione reductase and for the activity of NADPH-dependent thioredoxin system,[14,15] both are important in the protection of cells from oxidative damage. Therefore, ICDH may play an antioxidant role during oxidative stress. We recently reported that ICDH is involved in the supply of NADPH needed for GSH production against cytosolic and mitochondrial oxidative damage.[16,17] Hence, the damage of ICDH may result in the perturbation of the balance between oxidants and antioxidants and subsequently lead to a pro-oxidant condition.

In this report, the inactivation of ICDH mediated by lipid peroxidation products was examined in purified enzymes treated with MDA, HNE, and lipid hydroperoxide and in cultured human premonocytic U937 cells treated with 2,2′-azobis(2-amidinopropane) hydrochloride (AAPH), which induces lipid peroxidation in membrane.[18] The results indicate that lipid peroxidation may be an intermediary event in ICDH inactivation.

MATERIALS AND METHODS

Materials

RPMI 1640, fetal bovine serum (FBS), penicillin–streptomycin were obtained from GIBCO-BRL (Rockville, MD). Linoleic acid, soybean lipoxygenase, 1,1,3,3,-tetrahydroxypropane (TEP), AAPH, β-$NADP^+$, isocitrate, 2,4-dinitrophenylhydrazine (DNPH), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 5′-dithiobis-(2-nitrobenzoate) (DTNB), 8-anilino 1-napthalene sulfonic acid (ANSA), and mitochondrial $NADP^+$-dependent ICDH (IDPm) from pig heart were from Sigma Chemical Co. (St Louis, MO). Anti-human dinitrophenyl (DNP) antibody, anti-human HNE-Michael adduct antibody, and HNE was obtained from Calbiochem (La Jolla, CA). 2′,7′-Dichlorofluoroscin diacetate (DCFH-DA) was were purchased from Molecular Probes (Eugene, OR). In order to prepare recombinant $NADP^+$-dependent cytosolic ICDH (IDPc), *E. coli* transformed with pGEX-2λT containing an insert of mouse IDPc cDNA construct was grown and lysed, and the glutathione S-transferase (GST) fusion protein was purified on GSH-agarose as described elsewhere.[19] Antibody against IDPm was prepared from IDPm-immunized rabbit, and the antibody was purified by Protein A affinity chromatography.

Preparation of MDA and Linoleate Hydroperoxide

MDA was synthesized from TEP by heating at 50°C for 60 min as described.[20] The exact concentration of the stock solution of MDA was confirmed by measuring its absorbance at 245 nm ($\varepsilon = 13,700\,M^{-1}\,cm^{-1}$). To prepare lipid hydroperoxide (LOOH), freshly prepared ammonium salts of linoleic acid (13.5 μmol) were dispersed in 30 ml of 50 mM Tris–HCl buffer (pH 9.0) in a 250-ml Erlenmeyer flask equilibrated at 30°C. Oxygenation reactions were initiated by the addition of freshly prepared soybean lipoxygenase solution. After incubation, the mixture was applied in a C-18 reversed-phase sample preparation column (J.T. Baker). Fatty acid hydroperoxide was eluted with methyl formate.[21]

Cell Culture

Human premonocytic U937 cells (American Type Culture Collection, Rockville, MD) were grown in RPMI 1640 culture medium supplemented with 10% (v/v) FBS, penicillin (50 units/ml), and 50 μg/ml streptomycin at 37°C in a 5% CO_2–95% air humidified incubator.

Measurement of ICDH Activity

ICDH (6.5 μg) was added in a total volume of 1 ml containing 40 mM Tris buffer, pH 7.4, containing $NADP^+$ (2 mM), $MgCl_2$ (2 mM), isocitrate (5 mM). Activity of ICDH was measured by the production of NADPH at 340 nm at 25°C.[16] One unit of ICDH activity is defined as the amount of enzyme catalyzing the production of 1 μmol of NADPH/min.

For the determination of ICDH activities in mammalian cells, cells were collected at 1,000*g* for 10 min at 4°C and were washed once with cold PBS. Briefly, cells were homogenized with a Dounce homogenizer in sucrose buffer (0.32 M sucrose, 10 mM Tris–Cl, pH 7.4). Cell homogenates were centrifuged at 1,000*g* for 5 min and the supernatants further centrifuged at 15,000*g* for 30 min. The resulting supernatants were used as a cytosolic fraction to measure the activity of IDPc. The precipitates were washed twice with sucrose buffer to collect mitochondria pellet. The mitochondrial pellets were resuspended in 1X PBS containing 0.1% Triton-X100, disrupted by ultrasonication (4710 Series, Cole-Palmer, Chicago, IL) twice at 40% of maximum setting for 10 s, and centrifuged at 15,000*g* for 30 min. The supernatants were used to measure the activity of IDPm. The protein levels were determined by the method of Bradford using reagents purchased from Bio-Rad.

Determination of Protein Carbonyl Content

The carbonyl content of proteins was determined either spectrophotometric analysis with DNPH labeling or immunoblotting with anti-DNP antibody according to the method of Levine *et al.*[22] For the immunoblotting analysis, protein samples were precipitated with 20% TCA, and then treated for 1 h with 20 mM DNPH in 10% (v/v) trifluoroacetic acid at room temperature. After incubation, a neutralization solution (2 M Tris, 30% glycerol, 19% 2-mercaptoethanol) was added and further incubated at room temperature for 15 min. After SDS-PAGE of the derivatized proteins with 10% polyacrylamide gel, the separated proteins were probed with rabbit anti-DNP sera, used at a dilution of 1:1000.

Mass Spectrometry

Positive ion electrospray ionization mass spectrometry (ESI-MS) was performed on HP 1100 Series LC/MSD triple quadrupole mass spectrometer (Hewlett-Packard, Palo Alto, CA) equipped with an atmospheric pressure ion source. ICDH samples were subjected to gel filtration, and mixed with 0.1% trifluoroacetic acid. Aliquots of ICDH samples (5 μg protein) were directly applied to ESI-MS.

Thermal Inactivation

The heat stability of residual catalytic activity of partially oxidized ICDH (13 μg) was determined by incubation at 45°C in 40 mM HEPES, pH 7.0. At indicated times, an aliquot of this mixture was added to 1 ml of enzyme reaction mixture at 25°C to terminate the thermal inactivation. The residual activity was determined after the addition of the substrates.

Structural Analysis

For CD spectroscopy, samples of ICDH were desalted on Econo-Pac 10 DG column (Bio-Rad) equilibrated in 20 mM Tris buffer, pH 7.4, and fractions containing the protein were pooled. CD spectra were recorded on a temperature-controlled spectropolarimeter (JASCO, J-810). Spectra were recorded at 25°C in 0.05 cm quartz cells from 260 to 190 nm with protein concentrations of 0.05 mg/ml at a digital resolution of 0.5 nm, with scan speed of 5 nm/min for wavelength above and below 190 nm, respectively. Multiple spectra were recorded for duplicated samples. These spectra were averaged and corrected for baseline contribution from the buffer. Steady-state fluorescence measurements were performed on a Shimadzu RF-5301 PC spectrofluorophotometer with the sample compartment maintained at 22°C. A 150-W xenon source was used. The slit-width was fixed at 5 nm for excitation and emission. Unless otherwise stated, ICDH samples (15 μg) in 50 mM Tris–HCl, pH 7.4 were excited at 278 nm, and the emission was monitored between 300 and 400 nm. Each recorded spectrum was an average of three separate scans and was corrected for background fluorescence of the relevant control. ANSA (100 μM) was incubated with the various forms of ICDH in 25 mM potassium phosphate buffer, pH 7.0/50 mM KCl. The fluorescence emission spectra (excitation, 370 nm) of the different mixtures were monitored on spectrofluorometer. Binding of ANSA to the protein was evidenced by subtracting the emission spectrum of ANSA from that of ANSA in the presence of enzyme.

Measurement of Intracellular ROS

Intracellular peroxide production was measured using the oxidant-sensitive fluorescent probe DCFH-DA with confocal microscopy.[23] Cells were grown at 2×10^6 cells per 100 mm plate containing slide glass coated with poly-L-lysine and maintained in the growth medium for 24 h. Cells were treated with 10 μM DCFH-DA for 15 min and exposed to 50 mM AAPH for 15 min. Cells on the slide glass were washed with PBS and a cover glass was put on the slide glass. DCF fluorescence (excitation, 488 nm; emission, 520 nm) was imaged on a laser confocal scanning microscope (DM/R-TCS, Leica) coupled to a microscope (Leitz DM REB). The averages of DCF fluorescence intensity were calculated as described.[24]

Cellular NADPH and GSH Levels

NADPH was measured using the enzymatic cycling method as described by Zerez *et al.*[25] and expressed

as the ratio of NADPH to the total NADPH pool, [NADPH]/[NADPH + $NADP^+$]. The concentration of total GSH was determined by the rate of formation of 5-thio-2-nitrobenzoic acid at 412 nm ($\varepsilon = 1.36 \times 10^4\ M^{-1}\ cm^{-1}$) as the method described by Akerboom and Sies,[26] and GSSG was measured by the DTNB-GSSG reductase recycling assay after treating GSH with 2-vinylpyridine.[27] Total GSH level was measured in 0.1 M potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, 0.2 mg NADPH, 30 μg DTNB and 0.12 unit glutathione reductase. The GSSG level was measured in the same way as the total GSH level after treatment of 1 μl of 2-vinylpyridine and 3 μl of triethanolamine for 1 h.

Immunoblot Analysis

The proteins were resolved by SDS-PAGE on a 10% polyacrylamide gel. The separated proteins were then electrophoretically transferred to nitrocellulose membranes, and subsequently subjected to immunoblot analysis using appropriate antibodies. Immunoreactive antigen was then recognized by using horseradish peroxidase-labeled anti-rabbit IgG and an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech) or by using BCIP/NBT detecting system (Bio-Rad).

Immunoprecipitation

Mitochondrial fractions were cleared with Protein-A Sepharose (Amersham Pharmacia Biotech) for 1 h at 4°C. Supernatants were then incubated with rabbit polyclonal anti-IDPm (5 μg) for 12 h at 4°C followed by protein-A Sepharose incubation for 1 h at 4°C. Immunoprecipitated proteins were washed, separated by SDS-PAGE and visualized by staining with Coomassie Blue.

Replicates

Unless otherwise indicated, each result described in this paper is representative of at least three separate experiments.

RESULTS

ICDH was inactivated by MDA, HNE, and LHP in a time- and concentration-dependent manner, as shown in Fig. 1. Approximately 50% of the original activity was lost when the enzyme was exposed to 0.2 mM HNE, 3 mM MDA or 50 μM LOOH for 1 h at 37°C. The mouse liver IDPc was expressed as a fusion protein to GST, and it was purified as a recombinant protein, which was almost pure as estimated by SDS-PAGE (data not shown). As shown in Fig. 1D, a similar inactivation of recombinant IDPc by lipid peroxidation products was observed.

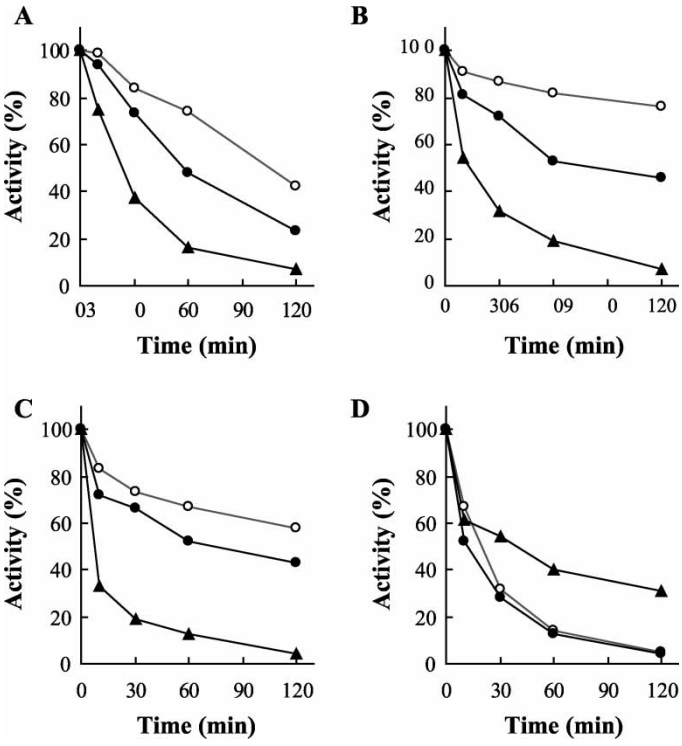

FIGURE 1 Time course of ICDH inactivation by lipid peroxidation products. IDPm was treated with (A) 0.1 (○), 0.2 (●), or 0.5 mM HNE (▲), (B) 1 (○), 3 (●), or 5 mM MDA (▲), (C) 20 (○), 50 (●), or 100 μM LOOH (▲). (D) IDPc was treated with 0.5 mM HNE (○), 5 mM MDA (●), or 50 μM LOOH (▲). Activities are given as a percentage of the control value. Means of triplicate assays are shown.

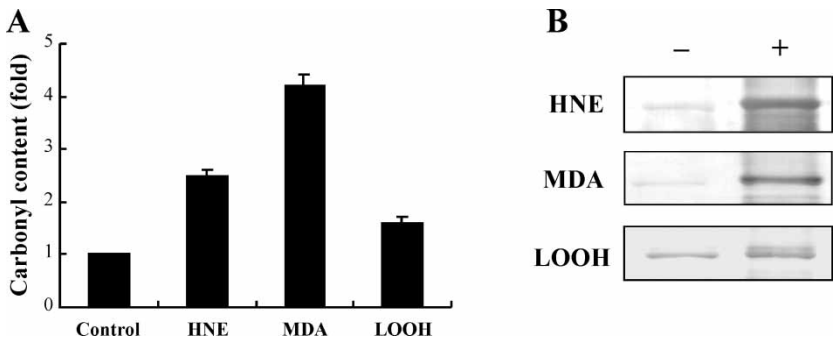

FIGURE 2 Protein carbonyl content of ICDH exposed to 0.5 mM HNE, 5 mM MDA, or 50 μM LOOH for 1 h at 37°C. (A) Protein carbonyls were measured by spectrophotometric method with the use of DNPH. Data are presented as means ± SD of four separate experiments. (B) Immunochemical analysis of carbonyl groups in ICDH. After incubation with lipid peroxidation products, samples derivatized with DNPH and DNPH-derivatized proteins were subjected to SDS-PAGE for immunoblotting with anti-DNP sera.

The carbonyl groups introduced into protein damaged by aldehydes and ROS react with DNPH to form a Schiff base, phenylhydrazine. Incubation of ICDH with lipid peroxidation products for 1 h at 37°C resulted in a significant increase in carbonyl groups (Fig. 2A). Immunoblotting results obtained from ICDH samples treated with lipid peroxidation products showed that an increase in the staining intensity of a 45 kDa band reflects the increase of carbonyl adducts (Fig. 2B).

Several oxidatively modified enzymes have been found to exhibit a greater heat sensitivity.[28] Increases in the rate at which modified versus native forms of the enzyme lose their remaining activity should reflect a decrease in the stability of this enzyme. As shown in Fig. 3A, ICDH modified by lipid peroxidation products lost its remaining activity at a faster rate, when incubated at 45°C, than did the native enzyme. To examine the secondary structure of the ICDH species after their modification with lipid peroxidation products, far UV-CD spectra of non-treated and treated ICDH were recorded and analyzed for specific elements of secondary structure. The CD spectrum of ICDH is very similar to that of the protein after modification with lipid peroxidation products, suggesting that the interaction of MDA, HNE, or LOOH with ICDH does not appreciably change the secondary structure of the protein. In an attempt to determine the effects of lipid peroxidation products on the conformation of ICDH, intrinsic fluorescence of the aromatic amino acids in each of the various forms of the enzyme were determined. Native ICDH exhibits a fluorescence emission spectrum typical for tryptophan

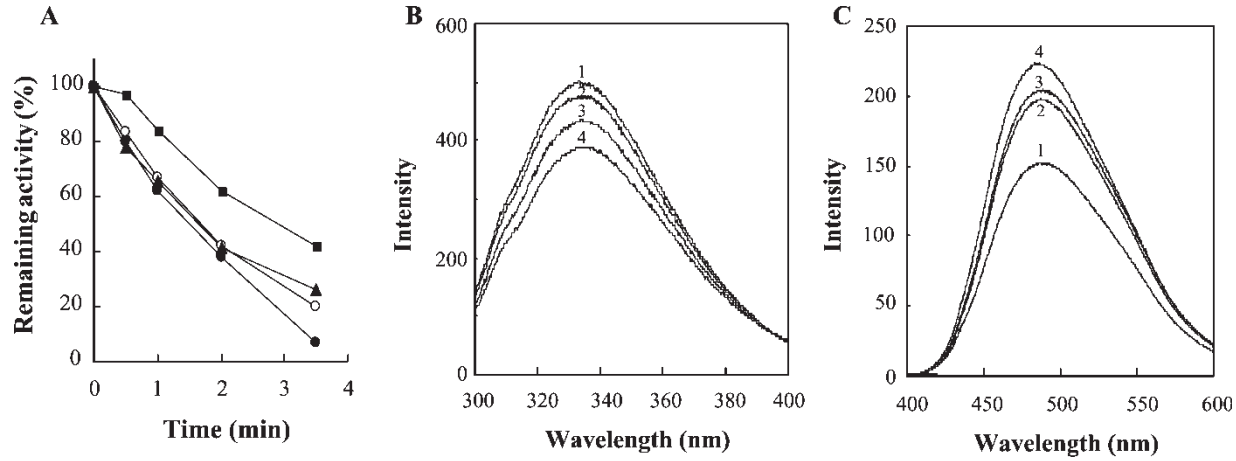

FIGURE 3 Structural changes in lipid peroxidation products-modified ICDH. (A) Heat inactivation of the modified ICDH. Enzyme previously inactivated by lipid peroxidation products to possess 50–60% of control activity was incubated at 45°C for an indicated time, and the residual activity was determined as described under "Materials and methods" section. ■, control; ○, 0.2 mM HNE; ●, 3 mM MDA; ▲, 50 μM LOOH. Means of triplicate assays are shown. (B) Steady-state emission spectra of the intrinsic fluorescence of native (line 1) and ICDH treated with 10, 20 and 50 μM LOOH (lines 2–4, respectively) for 1 h at 37°C were analyzed in a spectrofluorimeter. Spectra were obtained using an excitation wavelength of 278 nm and excitation and emission slits of 5 nm. All spectra were corrected for a protein blank emission. The fluorescence intensity is expressed in arbitrary units. (C) Spectrofluorometric analysis of ANSA binding to the modified ICDH. Emission spectra from 400 to 600 nm (excitation, 370 nm) of ANSA (100 μM) bound to native ICDH (line 1) and ICDH treated with 10, 20 and 50 μM LOOH (lines 2–4, respectively) for 1 h at 37°C. The increase in fluorescence intensity at 490 nm resulting from the binding of ANSA to the enzyme was determined by subtracting the emission spectrum of ANSA from that of ANSA in the presence of the different forms of the enzymes.

residues in proteins. Upon excitation of native ICDH at 278 nm, an emission spectrum with a maximum at 333 nm was observed. The fluorescence spectra of native and lipid peroxidation products-treated ICDH, normalized to the protein content, show that modified ICDH displays a dose-dependent decrease in quantum yield of the emission spectra and a blue shift of the maximum emission wavelength. The representative result with LOOH is shown in Fig. 3B. The fluorescence of free tryptophan was not influenced by lipid peroxidation products, indicating that subtle conformational changes may have occurred within lipid peroxidation product-treated ICDH in the vicinity of the aromatic residues. To reveal increases in flexibility of a partial unfolding of lipid peroxidation product-induced ICDH, the binding of the fluorescent probe ANSA was used to detect the accessibility of the hydrophobic regions on the protein. When ICDH was exposed to lipid peroxidation products, it bound the hydrophobic probe ANSA more efficiently than does the native protein. The representative result with LOOH is shown in Fig. 3C. Intensity of ANSA fluorescence was increased and the blue shift of maximum emission was seen in the LOOH-treated ICDH.

Because lipid peroxidation products readily inactivate ICDH *in vitro*, we examined ICDH activity in U937, a histiocytic lymphoma cell line, after treatment with 50 mM AAPH. AAPH undergoes spontaneous thermal decomposition producing carbon-centered radicals at a constant rate in the presence of oxygen; these radicals can attack membrane polyunsaturated fatty acids and initiate lipid peroxidation chain reaction.[18] Time-dependent decrease of both IDPc and IDPm activity in AAPH-treated cells was observed (Fig. 4A and B). To evaluate the effect of ICDH inactivation by AAPH on cellular redox status, we measured the cellular levels of NADPH and GSH as well as intracellular ROS generation. In cells treated with 50 mM AAPH for 1 h, the ratio of [NADPH]/[NADPH + $NADP^+$] was reduced by 21%, indicating that much less NADPH is present in AAPH-treated cells compared to control cells. One important parameter of GSH metabolism is the ratio of [GSSG]/[total GSH (GSHt)], which may reflect the efficiency of GSH turnover. The ratio of [GSSG]/[GSHt] in AAPH-treated cells was 2.3-fold higher than that of the control, indicating that GSSG in AAPH-treated cells was not reduced as efficiently as it was in the control cells. The effect of ICDH inactivation by AAPH on ROS generation was demonstrated by the relative intensity of DCF with confocal microscopy. DCF fluorescence intensity increased markedly in AAPH-treated cells compared with the control cells (Fig. 4C).

HNE is a major aldehyde product of lipid peroxidation and perhaps the most reactive. The HNE adduct of proteins has been widely used to evaluate the cellular damage induced by lipid peroxidation.[29] Immunoblot analysis of ICDH treated with 0.5 mM HNE (1 h, 37°C) *in vitro* revealed a 45 kDa band which immunoreactive with anti-HNE antibody (Fig. 5A). When ICDH was treated with 0.5 mM HNE (1 h, 37°C) and subjected to ESI-MS, molecular masses of unmodified and HNE-modified ICDH samples were 47,613 and 49,797 Da, respectively. The increased value corresponds to the incorporation of 14 HNE moieties to the parental protein. In order to identify the HNE-adduct of ICDH in cells, IDPm from control and AAPH-treated U937 cells were purified by immunoprecipitation. Western blot analysis of the purified IDPm with anti-HNE antibody showed immunoreactive bands at 45 kDa from AAPH-treated U937 cells but not from control cells (Fig. 5B).

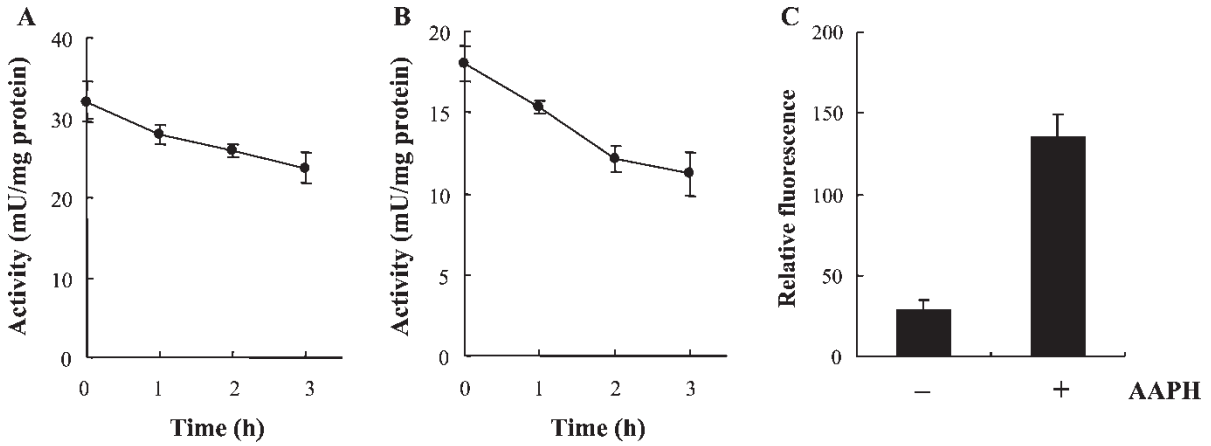

FIGURE 4 Inactivation of IDPc (A) and IDPm (B) in U937 cells exposed to 50 mM AAPH. Activities are given as a percentage of the control value. Data are presented as means ± SD of five separate experiments. (C) ROS generation in U937 cells treated with AAPH. Typical patterns of DCF fluorescence are presented for U937 cells untreated or treated with 50 mM AAPH for 15 min. Fluorescent images were obtained under laser confocal microscopy and the averages of fluorescence intensity were calculated as described.[28] Data are presented as means ± SD of triplicate experiments.

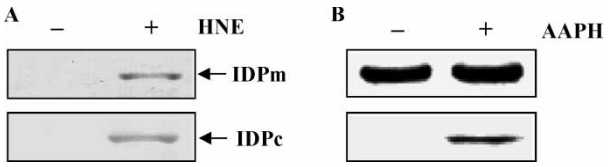

FIGURE 5 (A) Western blot analysis of HNE (0.5 mM)-modified ICDH. Proteins were separated on 10% SDS-PAGE, transferred to nitrocellulose membrane, and then subjected to immunoblot analysis using anti-HNE antibody. (B) IDPm was purified from control and AAPH-treated (50 mM, 3 h) U937 cells using immunoprecipitation with anti-IDPm antibody as described, then characterized by SDS-PAGE followed by immunoblotting. Purified IDPm was probed with anti-IDPm antibody (top) or anti-HNE antibody (bottom).

DISCUSSION

It has been proposed that lipid peroxidation is a continual process in living aerobic cells, is maintained at a low level, and can be prevented from entering into the autocatalytic phase by protective enzymes and antioxidants.[30] Chemical and physical agents that enhance membrane free radical reactions may accelerate this process beyond the capabilities of the protective systems, and thus cause widespread lipid peroxidation.[1] Lipid peroxidation of polyunsaturated fatty acid produces ROS and toxic aldehydes such as HNE and MDA. The ROS and aldehydes then contribute to the tissue damage that results by modifying critical biomolecules. In particular, the oxidative damage to antioxidant enzymes may lead to serious consequences. Therefore, the study of ICDH inactivation and its relationship to lipid peroxidation is relevant. Along with membrane-bound enzymes, soluble enzymes may also be damaged by lipid peroxidation products since it has been shown that oxidative stress induced phospholipase A_2, which may release lipid hydroperoxides from phospholipids.[31] Furthermore, aldehydes are more stable than free radical species and may more readily diffuse into cellular media, where they are available for facile reaction with various macromolecules.[32]

Although the exact mechanism for the inactivation of ICDH is not unequivocally elucidated, oxidation and structural alterations are major causes of enzyme inactivation. The reaction of reactive end-products of lipid peroxidation such as HNE, MDA, and secondary radicals with ICDH is likely to be important in the overall action of these toxic species. The reactions of MDA and HNE with proteins have received particular attention and were mainly found to involve the primary addition to nucleophilic residues such as Lys, His, or Cys.[33] ^{212}Lys, ^{315}His, and ^{387}Cys have been found to be essential for pig heart IDPm activity.[34,35] Therefore, we propose that Lys, His, and Cys located at the active site or at the cofactor binding site of IDPm are modified by aldehydes. In addition, secondary radicals may be involved in the oxidation of Cys results in the inactivation of the enzyme. Besides site-specific damage, global modification of susceptible amino acid residues outside the active site followed by a change of ICDH conformation may also lead to enzyme inhibition.

There are several lines of evidence obtained from the present study indicating that ICDH modified by lipid peroxidation products result in structural alterations. These findings are reflected in the changes in thermal stability and intrinsic tryptophan fluorescence and in binding of ANSA. However, the CD spectrum and, therefore, the secondary structure content of ICDH was not altered by the treatment with lipid peroxidation products, which suggests that only subtle, not drastic, conformational changes may occur in modified protein. ICDH modified by lipid peroxidation products appeared to be significantly more susceptible to heat inactivation than was native enzyme. It can be explained that lipid peroxidation products modify the structure of protein, such that the protein unfolds into a random conformation. The lower fluorescence quantum yield documents the alteration of the conformational integrity in lipid peroxidation products-modified ICDH. Modification of ICDH by lipid peroxidation products may lead to a slight disruption of protein structure, which is presumably responsible for the inactivation of enzymes, at least in part. Among the techniques aimed at following conformational changes of proteins, binding of the fluorescent probe ANSA has been used to detect the accessibility of the hydrophobic regions on protein upon increases in flexibility or partial unfolding. Binding can be easily monitored since it is accompanied by an increase in fluorescence and a blue shift of the emission maximum associated with the transfer of the ANSA from a hydrophilic to a hydrophobic environment.[28] A change in ANSA fluorescence at 490 nm in ICDH modified with lipid peroxidation products indicates conformational changes of protein.

Glucose 6-phosphate dehydrogenase, the first and rate-limiting enzyme of the pentose phosphate pathway, has long been regarded as the major enzyme to generate NADPH. In fact, the role of glucose 6-phosphate dehydrogenase in the cell response to oxidative stress is well established in yeast, in human erythrocytes, and in the mouse embryonic stem cells.[9,36,37] However, two other $NADP^+$-linked dehydrogenases, malic enzyme and IDPc, are also responsible for the generation of cytosolic NADPH.[38] Earlier study indicated that IDPc in the rat liver was 16- and 18-fold more active in producing NADPH than glucose 6-phosphate dehydrogenase and malic enzymes, respectively,[39] suggesting an important role of IDPc in the production of NADPH and eventually for the cellular defense against oxidative stress. Recently, IDPc that is preferentially expressed in bovine corneal epithelium has been identified. The role of this

enzyme in contributing to corneal transparency is likely attributed to its protective effect against UV radiation.[40] We also demonstrated that the control of cytosolic redox balance and oxidative damage is one of the primary functions of IDPc.[17] Mitochondria are the major source of O_2^- and highly susceptible to lipid peroxidation-mediated damage.[41] IDPm is a key enzyme in cellular defense against oxidative damage by supplying NADPH in the mitochondria, needed for the regeneration of mitochondrial GSH or thioredoxin. Elevation of mitochondrial NADPH and GSH by IDPm in turn suppressed the oxidative stress and concomitant ROS-mediated damage. It is well established that mitochondrial dysfunction is directly and indirectly involved in a variety of pathological states caused by genetic mutations as well as exogenous compounds or agents. Mitochondrial GSH becomes critically important against ROS-mediated damage because it not only functions as a potent antioxidant but is also required for the activities of mitochondrial glutathione peroxidase and mitochondrial phospholipid hydroperoxide glutathione peroxidase,[42] which removes mitochondrial peroxides. NADPH is a major source of reducing equivalents and cofactor for mitochondrial thioredoxin peroxidase family/peroxiredoxin family including peroxiredoxin III/protein SP-22[43–45] and peroxiredoxin V/AOEB166.[46]

The observed inactivation of ICDH in U937 cells by lipid peroxidation induced by AAPH leading to disturbances of integrity in the antioxidant defense mechanisms through the decrease in the generation of NADPH, an essential cofactor of GSH recycling. This may lead to pathological conditions associated with generation of ROS. In conclusion, the lipid peroxidation products-mediated damage to ICDH may result in the perturbation of cellular antioxidant defense mechanisms and subsequently lead to a prooxidant condition.

Acknowledgements

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